

AWARD NUMBER: W81XWH-14-1-0478

TITLE: Novel Therapeutic Approaches for the Treatment of Depression and Cognitive Deficits in a Rodent Model of Gulf War Veterans' Illness

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REPORT DATE: October 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

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1. REPORT DATE October 2015		2. REPORT TYPE Annual		3. DATES COVERED 29 Sep 2014 - 28 Sep 2015	
4. TITLE AND SUBTITLE Novel Therapeutic Approaches for the Treatment of Depression and Cognitive Deficits in a Rodent Model of Gulf War Veterans' Illness				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-14-1-0478	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Dr. Laxmikant S. Deshpande email: deshbandels@vcu.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) VIRGINIA COMMONWEALTH UNIVERSITY 912 W FRANKLIN ST RICHMOND, VA 23284-9040				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Approximately 1/3 rd of the returning veterans from the 1991 Persian Gulf War exhibit chronic multi-symptom illnesses that includes neurologic morbidities such as depression, anxiety and cognitive impairments. Amongst a host of causative factors, exposure to low-levels of the nerve gas Sarin is strongly implicated for expression of Gulf War Illness (GWI). Nerve agents similar to pesticides are organophosphate (OP) compounds. Here, we used various exposures (repeated low-dose to single high-dose) to OP diisopropyl fluorophosphate (DFP) over a 1 to 10-day period to approximate levels of Sarin exposure during the First Gulf War. We then tested the rats at 3-months post DFP exposure to reflect the current status of GW veterans. Using a battery of rodent behavioral assays, we observed the presence of symptoms of chronic depression, anxiety, and memory problems as characterized by increased immobility in the Forced Swim Test, anhedonia in the Sucrose Preference Test, anxiety in the Elevated Plus Maze, and spatial and recognition memory impairments in the Object Location/ Recognition Test. These rats also demonstrated neuronal damage in hippocampus, piriform cortex, amygdala, and thalamus. This animal model will help decipher molecular mechanisms for the expression of GWI neurological symptoms and screen effective treatment of GWIs.					
15. SUBJECT TERMS Gulf War Illness, Organophosphate, diisopropyl fluorophosphate (DFP), depression, anxiety, memory impairments, neuronal injury, Sprague-Dawley rats					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 31	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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1. Introduction

Approximately 175,000 to 250,000 of the returning veterans from the 1991 Persian Gulf War exhibit chronic multi-symptom illnesses that chiefly include chronic neurologic morbidities such as depression, anxiety and cognitive impairments. Amongst a host of causative factors, exposure to low levels of the nerve gas Sarin and Cyclosarin has been strongly implicated for expression of Gulf War Illness (GWI). There is evidence from civilian population that exposure to OPs such as in agricultural workers and nerve agent exposures seen in the survivors and first-responders of the Tokyo subway Sarin gas attack suffer from chronic psychiatric problems that are similar to GWI neurological symptoms. Nerve agents mechanistically resemble pesticides and chemically are organophosphate (OP) compounds. Given this unique chemical profile, OPs are ideal to study the effects of nerve agents and develop rodent models of GWI in civilian laboratories. In this study, we used various dose (repeated low-dose to single high-dose) exposure to OP diisopropyl fluorophosphate (DFP) over a 1 to 10-day period to approximate levels of Sarin exposures during the Persian Gulf War. We then tested the rats at 3-months post DFP exposures to reflect the current status of GW veterans. Using a battery of behavioral assays, we observed the presence of symptoms of chronic depression, anxiety and memory problems as characterized by increased immobility in the Forced Swim Test, anhedonia in the Sucrose Preference Test, anxiety in the Elevated Plus Maze, and spatial and recognition memory impairments in the Object Location/ Recognition Tests respectively. These rats also demonstrated significant neuronal damage in hippocampus, piriform cortex, amygdala and thalamus. These brain areas are integral to normal physiological function and structural damage in some of these brain areas particularly hippocampus have been reported in GW veterans. Given that OP exposure is considered a leading cause of GWI related morbidities, this animal model will be ideally suited to study underlying molecular mechanisms for the expression of GWI neurological symptoms and identify drugs for the effective treatment of GWIs.

2. Keywords

Gulf War Illness, Organophosphate, diisopropyl fluorophosphate (DFP), depression, anxiety, memory impairments, neuronal injury, Sprague-Dawley rats

3. Accomplishments:

The following lists the accomplishments from our project during the first year period (2014-2015) of this grant.

3.1 What were the major goals of the project?

As listed in the SOW, the following were the major goals of the projects for year 1. We have accomplished all these goals. A detailed description of each of these milestones is in section 3.2

- A. Obtain IACUC and ACURO approval for animal protocol
- B. Test OP dosing regimens to mimic OP exposure levels seen by GW veterans
- C. Behavioral screening of the DFP exposed animals for anxiety, depression and cognitive deficits at 3-month following the initial exposure
- D. Histological screening of the DFP exposed rats for assessing neuronal damage

Specific Aim for year 1: To develop a rat model of OP exposure mimicking different OP levels seen by Gulf War veterans and determine development of associated chronic GWI morbidities		
Major Task 1: Test various dosing regimen with OPs to mimic the different OP exposure levels seen by Gulf War veterans	Months	% Completed
Subtask 1: Obtain IACUC and ACURO approvals	1-2	100%
Subtask 2: Assessment of acute mortality and sub-chronic general behavior in rodents following DFP exposures (0.1 mg/kg, s.c., 10-days, 0.5 mg/kg, s.c., 5-days and 4 mg/kg, s.c., 1-day).	2-3	100%
<i>Milestone(s) Achieved: Necessary approvals from IACUC and ACURO obtained before experimentation. Identification of three different dosing schedules for DFP to mimic Gulf War veterans OP exposure levels.</i>		
Major Task 2: Histological screening of the DFP exposed animals for assessing hippocampal damage	Months	% Completed
Subtask 1: Assessment of neuronal damage in rats exposed to low-dose DFP (0.1 mg/kg, s.c., 10-days)	3-6	100%
Subtask 2: Assessment of neuronal damage in rats exposed to moderate-dose DFP (0.5 mg/kg, s.c., 5-days)	3-6	100%
Subtask 3: Assessment of neuronal damage in rats exposed to high-dose DFP (4 mg/kg, s.c., 1-day)	3-6	100%
<i>Milestone(s) Achieved: Assessment of neuronal injury in brain area responsible for related neurological function in rats exposed to various DFP dose levels.</i>		
Major Task 3: Behavioral screening of the DFP exposed animals for anxiety (elevated plus maze), depression (sucrose preference test and forced swim test) and cognitive deficits (novel object recognition/ location) at 3-month following the initial exposure	Months	% Completed
Subtask 1: Assessment of anxiety, depression and cognitive deficits in rats exposed to low-dose DFP (0.1 mg/kg, s.c., 10-days)	7-12	100%
Subtask 2: Assessment of anxiety, depression and cognitive deficits in rats exposed to moderate-dose DFP (0.5 mg/kg, s.c., 5-days)	7-12	100%
Subtask 3: Assessment of anxiety, depression and cognitive deficits in rats exposed to high-dose DFP (4 mg/kg, s.c., 1-day)	7-12	100%
<i>Milestone(s) Achieved: Assessment of underlying anxiety, depression and cognitive deficits in rats exposed to various DFP dose levels using a battery of rodent behavioral assays. Data analysis. Research findings communicated both in abstract and manuscript form.</i>		

3.2 What was accomplished under these goals?

A. *Protocol approvals:*

We obtained approvals from VCU IACUC and ACURO committees for our animal protocol in the anticipated 1-month time period following grant approval. Our animal protocol was reviewed again and was approved without any changes for 3-more years by IACUC. The new expiration date of the animal protocol is 06/09/2018. Since then, we have submitted the renewed IACUC protocol to ACURO for review. We are not expecting any major queries from ACURO since no changes were made to the earlier protocol that was approved by ACURO last year.

B. *DFP exposure protocols:*

There are several confounding factors attributed to development of Gulf War Illness (GWI), including exposure to depleted uranium from tanks and body armor, prophylactic use of pyridostigmine bromide tablets, heavy use of insect repellants such as DEET and permethrin, smoke from oil-well fires, and dust particulate matter among others [1-3]. Newly assembled epidemiological, meteorological and intelligence data now indicate soldiers were exposed to organophosphate (OP) nerve agents Sarin and Cyclosarin from fallout released from demolitions of the ammunition dump at Khamisiyah, Iraq [4-7]. To mimic this OP exposure, we chose diisopropyl fluorophosphate (DFP) an OP compound that is used in civilian laboratories as a surrogate nerve gas agent [8-11]. Similar to Sarin, DFP is also an irreversible inhibitor of the enzyme acetylcholinesterase (AChE). While there are no correct estimates available for levels of nerve gas exposure [12], it is generally believed that there were no troops in the immediate vicinity of “first-noticeable effect” zone. The majority of plume exposed soldiers were in “low-level hazard” zone [13]. In an attempt to develop an OP-based rodent model of GWI neurological morbidities, we used DFP dose that were 1/20th, 1/5th and 2x the LD₅₀ estimates [14] to mimic various levels of GW nerve agent exposures. The goal of these studies was to conduct assessment of acute mortality and sub-chronic general behavior in rodents following DFP administrations (0.1 mg/kg, s.c., 10-days, 0.5 mg/kg, s.c., 5-days and 4 mg/kg, s.c., 1-day).

DFP (catalog # D0879) was prepared fresh daily by dissolving in ice-cold phosphate buffered saline just before the exposure. Rats were injected with DFP (dosing protocols as above), while control rats received DFP vehicle injections for the same period. Animal health including weight measurements were assessed every day during the exposure and for the next seven days following the end of DFP injections.

Low dose DFP injections (0.1 mg/kg, s.c., 10-days) were very well-tolerated by rats. No signs of cholinergic hyperactivation were observed in these animals. Mortality was none. No difference in weight gain dynamics was observed in treated vs. non-treated rats. Moderate dose DFP injections (0.5 mg/kg, s.c., 5-days) were generally tolerated by the rats. Again no major signs of cholinergic activation were observed, particularly during the first 4-days of DFP administration. Few rats displayed lacrimation and mild tremors on the 5th day of exposure but these symptoms were resolved without any intervention by the end of the day. No significant differences were observed in the weight gain dynamics between the controls and DFP-exposed rats. There were no visual signs of pain and discomfort such as hunched posture, poor grooming, porphyrin around eyes or nose in DFP exposed rats. No mortality was observed following this DFP dosing schedule. The high-dose DFP corresponds to the scenario of using the Mark-I kits (atropine, 2-PAM and diazepam) following Sarin exposure. Again, so far there are no reports of soldiers using this kit following OP exposure but they did carry these kits and it is worthwhile to

investigate a scenario when these buddy kit would have been used. Rats were injected with high-dose DFP (4 mg/kg, s.c., 1-day) followed by atropine sulfate (2 mg/kg, i.p.) and 2-PAM (25 mg/kg, i.m.) one minute later. At this high-dose, DFP had a very quick onset of action. Within 2-3 minutes of injections, hypercholinergic effects were observed characterized by salivation, urination, defecation, tremors and wet-dog shakes. Within 5-8 minutes seizures were observed and characterized by myoclonic jerks and convulsions that quickly evolved into non-stop seizure activity that did not abate until we initiated anticonvulsant treatment with sequential diazepam injections. Mortality following high-dose DFP exposure and subsequent rescue using the atropine + 2-PAM + diazepam therapy was approximately 10%.

C. Behavioral screening:

Amongst the GWI morbidities, the neurological deficits such as chronic depression, anxiety and memory impairments are pre-dominant ones. To investigate whether such DFP exposures would lead to expression of psychiatric abnormalities, we conducted a battery of rodent behavioral assays to identify symptoms of depression, anxiety and cognitive deficits at 3-months following various DFP exposures to closely represent the current status of GW veterans. Testing was carried out in a quiet, dimly lit room between 0800 to 1400 hrs. Behavioral testing moved from the least stressful to most stressful tasks. Thus, rats were first subjected to sucrose preference test followed by object recognition/ location test, then elevated plus maze and finally forced swim test. No two tests were carried out on the same day.

Depression is a complex psychological phenomenon and as such is difficult to analyze using a single test (Overstreet, 2012). For identifying depressive symptoms the Forced Swim Test (FST) that models despair along with the Sucrose Preference Test (SPT) that signifies anhedonia were used. Anxiety was tested using Elevated Plus Maze (EPM) paradigm. Together, the helplessness, despair, lack of feeling pleasure and anxiety constitute the symptoms of depression-like state in rats. Like depression, assessment of memory is a complex behavioral task. Memory was tested using Novel Object Recognition (NOR) test, which is similar to the human delayed non-match to sample task and assess pre-frontal function. Spatial memory was tested using the Object Location Test (OLT) and assess hippocampus dependent memory function. These tests are described below:

Forced Swim Test (FST)

Porsolt's modified FST was used to assess behavioral despair [15-17]. Briefly, animals were forced to swim by being placed in a glass cylindrical chamber (46cm H x 30cm D) filled with water (30 cm height, 25°C). Two swimming sessions were carried out with an initial 15 min 'pre-test' followed by a 5 min 'test' after 24 h. Swimming sessions were recorded for off-line analysis. Active (swimming, climbing, diving) and passive (immobility) behavior was evaluated by 2 reviewers blinded to the treatment conditions. Immobility (primary outcome) was defined as the period during which the animal floats in the water making only those movements necessary to keep its head above water. The tank was emptied and thoroughly cleaned for every rat to be tested in a session.

Sucrose Preference Test (SPT)

This test measures hedonia (pleasure-seeking) or lack of it (anhedonia) by monitoring a rat's preference to sucrose-laced water [15,16]. Briefly, rats were habituated to having two bottles in the cage lid for three days. The bottles were fitted with ball-bearing sipper tubes that

prevented fluids from leaking. Following this acclimation, rats had the free choice of either drinking the 1% sucrose solution or plain water for a period of 2 days. Water and sucrose solution intake was measured daily, and the positions of two bottles were switched daily to reduce any confounding effects produced by a side bias. Sucrose preference was calculated as a percentage of the volume of sucrose intake over the total volume of fluid intake and averaged over the 2 days of testing. Reviewers were blinded to treatment conditions. A spill-cage without rat was also employed using bottles and sipper tubes from the same batch as test cages. Measurement errors were ± 2 ml.

Elevated Plus Maze (EPM)

This test assesses anxiety by taking into account the innate behavior of rats to prefer dark enclosed spaces over bright open spaces [16,18]. The maze (Med Associates Inc., St. Albans, VT) was made of black polyvinyl chloride and consisted of four arms, 50 cm long x 10 cm wide, connected by a central square, 10 x 10 cm: two open without walls and two closed by 31-cm-high walls. All arms were attached to sturdy metal legs; the maze was elevated 55 cm above the floor level and was set in a dimly lit room. A video camera was suspended above the maze to record the rat movements for analysis. A video-tracking system (Noldus Ethovision XT 11) was used to automatically collect behavioral data. The procedure consisted of placing the rats at the junction of the open and closed arms, the center of the maze, facing the open arm opposite to where the experimenter was. The video-tracking system was started after the animal was placed in the maze so that the behavior of each animal was consistently recorded for 5 min. At the end of the 5 min test session, the rat was removed from the plus maze and returned to its home cage. The maze was cleaned with 70% ethanol and air-dried to remove any scent traces and allowed to dry completely before introducing the next animal in the arena. Time spent and entries made in the various arms of EPM were calculated.

Novel Object Recognition (NOR)

Briefly, rats were placed in black Perspex box 60 x 90 x 50 cm in a dimly illuminated and quite animal behavior testing room. Rats were habituated individually, by allowing them to explore the box for 15-min per session for 3 days. The arena was cleaned with a 70% ethanol solution and dried completely in between each subject so as to eliminate any potential odor cues left by previous subjects. On the fourth day, in the sample phase, two identical objects (A) were placed in opposite corners of the box, 20 cm from the wall. A rat was allowed to explore for 3-min, and then it was removed from the box and returned to its home cage. In the choice phase (3-h later), one familiar object was replaced by a novel object (B), and the rat was allowed to explore for 2-min. Objects were similar in size and emotionally neutral, but varied for shape, color and texture. Behavior was recorded by a digital camera mounted above the experimental box. Direct contacts included any contact with mouth, nose or paw and did not include contacts that were accidental (backing or bumping into the object). Also, standing, sitting or leaning on the object was not scored as object interaction. We determined a discrimination ratio, which is a difference in the exploration time divided by the total time spent exploring the two objects in the choice phase: $([B - A] / [B + A])$. A time-dependent decline in preference for novel object is likely reflective of memory decline for familiar object.

Object Location Test (OLT)

This test assesses place recognition memory (spatial memory) by calculating the preference of the rat to explore an object that has been moved to a new location. Briefly, rats were placed in a black Perspex box 90 x 60 x 50 cm in a dimly illuminated and quiet animal behavior testing room. Rats were habituated individually, by allowing them to explore the empty box for 10-min per session for 2 days. The arena was cleaned with a 70% ethanol solution and dried completely in between each subject so as to eliminate any potential odor cues left by previous subjects. On the third day, in the sample phase, two identical objects were placed in opposite corners of the box, 20 cm from the wall. A rat was allowed to explore for 3-min, and then it was removed from the box and returned to its home cage. In the choice phase (1-h later), one of the objects was moved to a novel location, and the rat was allowed to explore for 2-min. Objects were similar in size and emotionally neutral. A video-tracking system (Noldus Ethovision XT 11) was used to automatically collect behavioral data. Direct contacts included any contact with mouth, nose or paw and did not include contacts that were accidental (backing or bumping into the object). Also, standing, sitting or leaning on the object was not scored as object interaction. A rat is considered to be exploring an object when its nose is within 2 cm of the object. Time spent exploring the object at novel location versus the object remaining in the familiar location was calculated for each group. A place discrimination index was calculated as the percentage of time spent with the object at novel location/the total time spent in exploring both the objects. [16,19,20].

D. Histological analysis for assessing neuronal damage:

To study the effects of various DFP exposures on brain damage, we assessed neuronal injury in major brain areas including hippocampus (dentate gyrus, CA1), cortices (parietal, peri-rhinal, piriform), thalamus, and amygdala. Damage to these areas is associated with expression of behavioral and neurological morbidities and therefore may underlie development of GWI related neuropsychiatric symptoms. For example, hippocampus is essential in memory functioning [21] and plays a major role in pathophysiology of depression [22]. Studies have shown hippocampal dysfunction in Gulf War veterans using both imaging and neuropsychological testing [23-26]. Chronic hippocampal perfusion dysfunction [27] and smaller hippocampal volume [28] has been observed in GW veterans. Reduced gray matter, white matter and hippocampal subfields have also been reported in GW veterans suspected with Sarin and Cyclosarin exposure [23,24,29]. Animal models of GWI have also demonstrated hippocampal neuronal loss, reduced neurogenesis, inflammation, and reduced synaptic transmission underlying the expression of anxiety, depression, mood and memory deficits [11,20,30-34]. To assess neuronal injury brain sections from animals injected with DFP or vehicle were labeled with FJC [9,35] using procedures described below.

Fluoro-Jade staining:

Animals were sacrificed 48-h following DFP exposure. Briefly, deep anesthesia was induced in rats with ketamine/ xylazine (75mg/kg/7.5mg/kg i.p.) mixture. Anesthetized animals were flushed transcardially with saline and perfused with 4% paraformaldehyde in a 100 mM sodium phosphate buffer (pH 7.4). Fixed brains were removed and post-fixed in 4% paraformaldehyde/phosphate buffer overnight, cryoprotected in 30% sucrose/phosphate buffer (pH 7.4) (48 h), flash frozen in isopentane and stored at -80°C until used for sectioning. Coronal sections (40 µm) were cut on a cryostat (Leica Microsystems, Wetzlar, Germany) and mounted onto microscope slides (Trubond 380; Tru Scientific LLC, Bellingham, WA). Slides were dried

in a desiccant chamber at 55°C for 30 min prior to staining. Slides were first incubated in a solution of 1% NaOH in 80% ethanol for 5 minutes followed by hydration in a 70% ethanol and then ddH₂O for 2 minutes each. Slides were then incubated in a 0.06% KMnO₄ solution for 10 min followed by washing in ddH₂O for 2 min. Slides were then stained in a 0.0004% Fluoro-Jade C (FJC) solution in 0.1% acetic acid for 20 min (Deshpande et al., 2014a; Li et al., 2011b). Stained slides underwent 3x washes in ddH₂O for 2 min each and then dried in a desiccant chamber at 55°C for 30 min. Stained slides were then cleared with xylene for 5 min and cover slipped with DPX mounting agent. Stained sections were evaluated with a fluorescent IX-70 inverted microscope with a 20X (UApo 340, 0.7 n.a., water) objective (Olympus America, Center Valley, PA) and excitation/emission filters for visualization of FITC. Greyscale digital images (1324x1024, 16-bit, 1x1 binning) of FJC staining for hippocampus were acquired with a Hamamatsu ORCA-ER camera (Hamamatsu Photonics, Japan).

Data analysis

Data were analyzed and graphs plotted using the SigmaPlot 12.5 software (SPSS Inc, Chicago, IL). All the data that passed the normality test was further subjected to t-test. A value of $p < 0.05$ was considered significant for all data analyses. Analysis of digital images to count FJC positive cell staining was carried out with ImageJ (U. S. National Institutes of Health, Bethesda, MD) by thresholding for specific stain and obtaining positive cell counts using the particle analysis component (size range in pixel: 25-1000). All parameters for digital acquisition and analysis of staining remained constant throughout. Representative digital images were processed with Adobe Photoshop (Adobe Systems Inc., San Jose, CA).

E. Results

Performance on FST

The FST was an effective test in evaluating the presence of a despair-like state in the DFP exposed rats (Figure 1). High-dose DFP rats (4mg/kg, 1-day) subjected to FST exhibited increased immobility time (83.03 ± 8.5 s, $n = 11$) indicative of a despair-like state that was significantly higher than age matched controls (35.46 ± 3.8 s, $n = 9$, $p < 0.05$). Moderate-dose DFP rats (0.5 mg/kg, 5-days) subjected to the FST also exhibited increased immobility time (78.7 ± 11.5 s, $n = 11$) that was significantly higher compared to the immobility time (37.7 ± 6.5 s) in age matched control rats ($p < 0.01$, $n = 9$). No significant differences in the immobility time were observed between low-dose DFP rats (0.1 mg/kg, 10-days, $n = 9$) and the age-matched control rats (40.6 ± 3.5 s). To avoid control data redundancy and improve readability, only one averaged control group is plotted in the bar-graph (Fig. 1).

Performance on SPT

DFP exposed rats also displayed absence of preferential sucrose consumption on SPT (Figure 2). High-dose DFP rats (4 mg/kg, 1-day, $n = 15$) consumed significantly less sucrose water indicating anhedonia-like condition ($58.40 \pm 5.9\%$ sucrose preference in DFP rats Vs $82.66 \pm 6.5\%$ in control rats, Fig. 2D). Moderate-dose DFP rats (0.5 mg/kg, 5-days, $n = 15$) also consumed $53.2 \pm 4.8\%$ sucrose-laced water and $46.8 \pm 5.2\%$ of non-sweetened water (Fig. 2C). Low-dose DFP rats (0.1 mg/kg, 10-days, $n = 15$) also displayed relatively moderate symptoms of anhedonia by preferring sucrose-water $60.54 \pm 5.8\%$ over non-sweetened water $39.46 \pm 7.5\%$ (Fig. 2B). In contrast, age-matched control rats overwhelmingly preferred sucrose water ($74.1 \pm$

4.1 %) over non-sweetened water ($25.9 \pm 5.2\%$) (Fig. 2B, C, D). This indicates presence of anhedonia in DFP exposed rats ($p < 0.01$, $n = 12$). No differences were found between total fluid consumption and fluid consumed on right vs. left amongst the control and various DFP exposed groups ($p > 0.5$, Fig. 2A). To avoid control data redundancy and improve readability, only one averaged control group is plotted in the bar-graph (Fig. 2A).

Performance on EPM

DFP exposed rats also displayed symptoms of anxiety when subjected to EPM test (Figure 3). High-dose DFP rats (4 mg/kg, 1-day, Fig. 3A, B) displayed increased anxiety as characterized by significantly lower performance in the open arm of the EPM (time in open arm: $18.9 \pm 4.5\%$ in controls vs $4.2 \pm 1.5\%$ in DFP rats) and also made significantly less entries in the open arm ($9.4 \pm 2.2\%$ vs. 39.8 ± 5.6), further indicating the presence of symptoms of anxiety. Similarly, moderate-dose DFP rats (0.5 mg/kg, 5-days, Fig. 3A, B) spent significantly less time ($9.4 \pm 2.2\%$ vs. $29.3 \pm 3.7\%$) and made significantly less entries ($12.2 \pm 4.8\%$ vs. $37.1 \pm 4.5\%$) in the open-arm of EPM compared to age-matched control rats ($p < 0.05$, $n = 11$). Low-dose DFP rats (0.1 mg/kg, 10-days, Fig. 3A, B) also spend significantly less time in open arm of EPM ($5.28 \pm 1.7\%$ vs $12.86 \pm 1.9\%$ in controls). However, no significant differences were found between numbers of entries made in the open arm between the two groups (28.02 ± 4.9 in DFP rats vs $30.92 \pm 5.7\%$ in controls). To investigate whether these differences in open-arm behavior were not due to global differences in exploratory or locomotor activity, we also measured the distance travelled and total arm entries. No significant differences were observed in these two parameters between the DFP exposed rats and age-matched control rats in these groups ($p > 0.5$, $n = 11$). To avoid control data redundancy and improve readability, only one averaged control group is plotted in the bar-graph (Fig. 3C, D).

Performance on NOR

The NOR test revealed deficits in recognition memory in DFP exposed rats (Figure 4). In the choice phase of NOR, high-dose DFP exposed rats (4 mg/kg, 1-day) spent more time exploring the old object compared to the new object indicating that these rats did not remember the familiar object. These rats exhibited a discrimination ratio of 0.28 ± 0.09 indicative of impaired recognition memory that was significantly lower compared to age matched control rats (0.76 ± 0.07 , $n = 15$, $p < 0.05$). Similarly, moderate-dose DFP rats (0.5 mg/kg, 5-days) exhibited a discrimination ratio (0.42 ± 0.18) indicative of impaired recognition memory that was significantly lower compared to age matched control rats ($p < 0.01$, $n = 15$). Further, low-dose DFP exposed rats (0.1 mg/kg, 10-days) exhibited a lower discrimination ratio (0.58 ± 0.11) that was lower but not significantly different from age-matched control rats.

Age-matched control rats always spent more time exploring the novel object across all the three groups indicating that these rats remembered the familiar object. Moreover, in the sample phase (3-h earlier) no significant exploratory preference was found between the two groups and rats from both the group spent similar time exploring the two objects ($p > 0.5$, $n = 15$). To avoid control data redundancy and improve readability, only one averaged control group is plotted in the bar-graph (Fig. 4A, B).

Performance on OLT

DFP exposed rats displayed significant deficits in spatial memory in OLT (Figure 5). In the test phase of OLT, age-matched control rats spent more time exploring the object at the new location (B) versus the object at the old place (A) (70.87% vs 29.13%, respectively), indicating that these rats remembered the earlier location (Fig. 5A, B). In contrast, high-dose DFP exposed rats (4 mg/kg, 1-day) showed little preference when the object was at the novel location (B) and spent less time exploring object at novel location B (59.7% vs 40.3%, respectively), indicating that these rats displayed spatial memory impairments. Similarly, moderate-dose DFP exposed rats (0.5 mg/kg, 5-days) also showed little preference when the object was at the novel location (B) and spent almost equal time exploring object at both locations (48.7% vs 51.3%, respectively), indicating that these rats displayed spatial memory impairments. Calculating the place discrimination index revealed impaired place recognition memory in the DFP exposed rats ($p < 0.01$, $n = 15$). In contrast, no significant spatial memory impairments were observed between low-dose DFP exposed (0.1 mg/kg, 10-days) and age-matched control rats (Fig. 5B). No significant differences were observed between distance travelled and mean velocity amongst the groups ($p > 0.5$, $n = 15$). To avoid control data redundancy and improve readability, only one averaged control group is plotted in the bar-graph (Fig. 5A, C, D).

Histological Observations:

Significant brain damage was observed in various brain regions in DFP exposed rats (Figure 6). To assess neuronal injury in OP exposed animals, brain sections from animals injected with DFP or vehicle were labeled with Fluoro-Jade C (FJC). Across all brain regions examined, there was negligible FJC labeling in brain sections obtained from vehicle controls (Fig. 6A, B, $n = 5$). In contrast, after high-dose DFP exposure (4 mg/kg, s.c., 1-day, $n = 6$), FJC-positive cells were observed in select regions throughout the forebrain. Within the hippocampus, FJC-positive staining was observed in the polymorphic layer and along the hilus/granule cell border of the dentate gyrus and CA1 region. Additionally, FJC-positive stained neurons were observed throughout layers II and III of the parietal cortex. Distinct FJC staining was also observed in piriform cortex and perirhinal cortex. Further, amygdala and thalamus were also stained positive for FJC. Representative images for neuronal damage in various brain regions following high-dose DFP exposure (4 mg/kg, s.c., 1-day) are shown in Fig. 6A. The quantification of the neuronal injury expressed as FJC positive cells was conducted as described above and is shown in Fig. 6B. Next we assessed neuronal damage following moderate-dose DFP exposure (0.5 mg/kg, s.c., 5-days, $n = 6$). Vehicle treated rats displayed no FJC staining in brain sections. However, neuronal damage particularly in hippocampus as characterized by presence of diffused FJC-positive staining in the polymorphic layer and along the hilus/granule cell border of the dentate gyrus was observed (Fig. 6C). FJC staining was not observed in other brain regions such as cortex, amygdala and thalamus. Quantitative analysis revealed presence of 2.1 ± 0.22 FJC positive cells/ $100 \mu\text{M}^2$ area in hilus of moderate-dose DFP exposed rats. Finally the low dose DFP (0.1 mg/kg, s.c., 10-days, $n = 6$) also produced a negligible FJC staining, which was comparable to the control rats. Any FJC staining, if any, was mostly restricted to the hilus within the dentate gyrus. Other brain regions including the cortices, amygdala and thalamus were found to be negative for FJC staining. Representative staining images are shown in Fig. 6C.

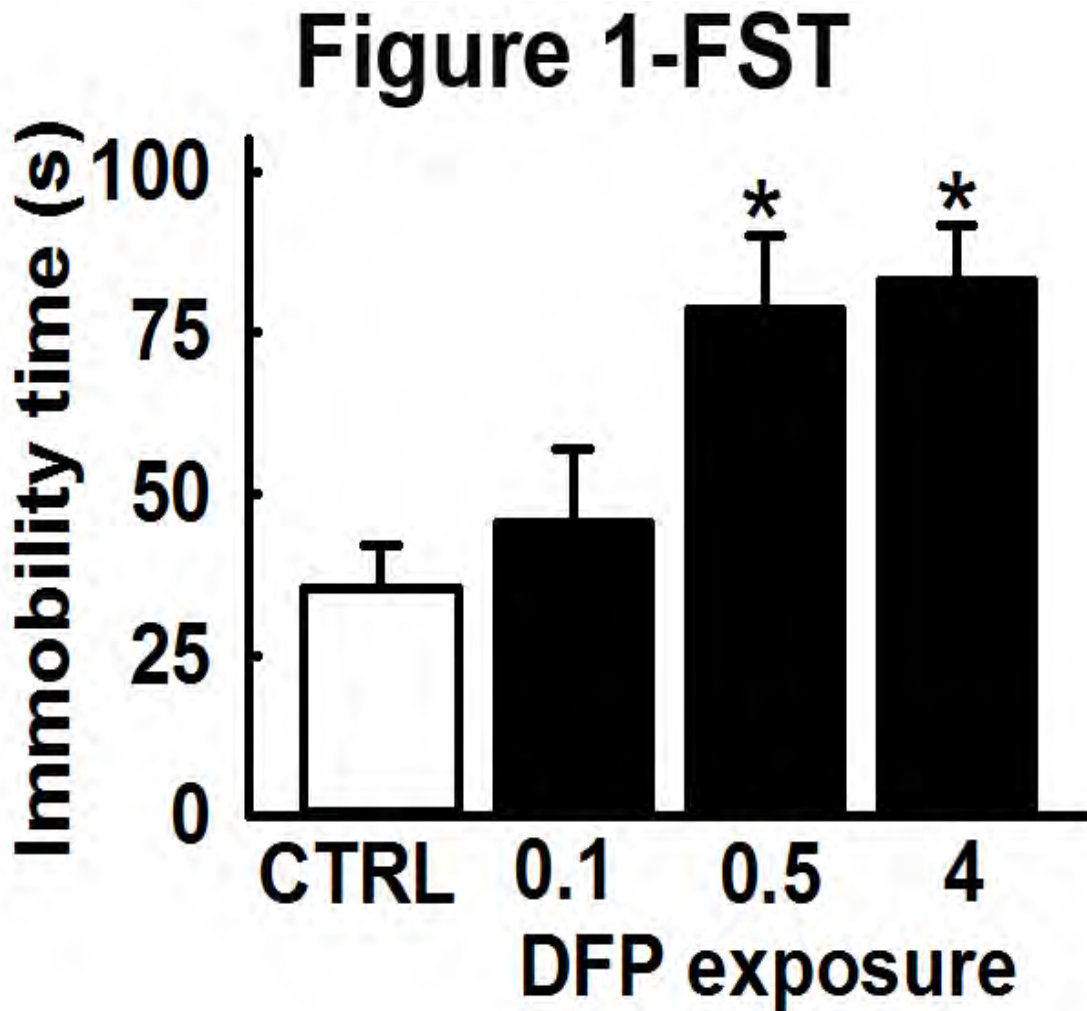


Figure 1. Increased immobility time in DFP exposed rats during FST. The immobility time in DFP exposed rats (0.5 and 4 mg/kg group) was significantly higher compared to age matched control rats. Data expressed as mean \pm SEM, * p <0.05, t-test, n = 9-11 rats.

Figure 2- SPT

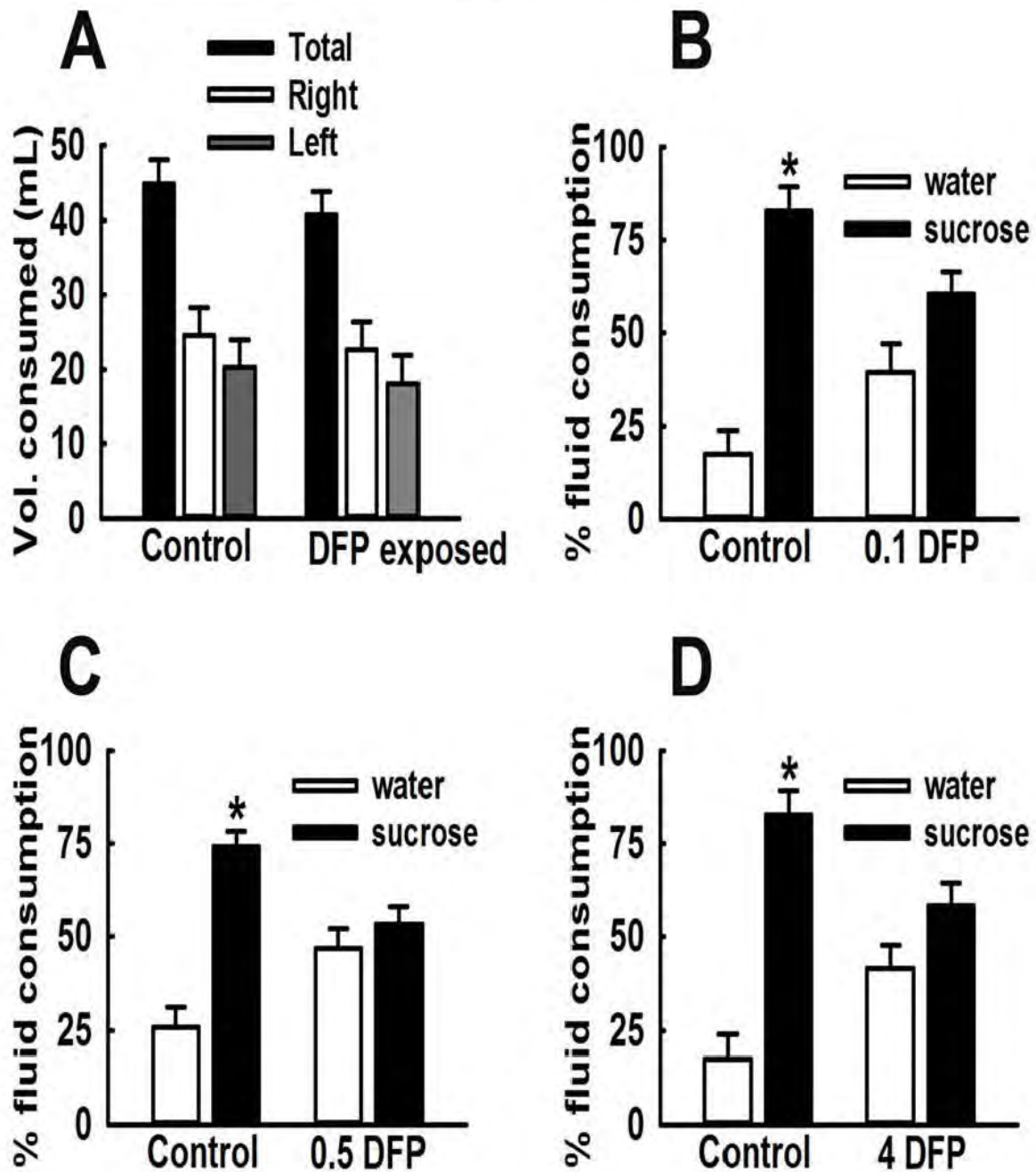


Figure 2. Loss of sucrose consumption preference in DFP exposed rats on SPT. Control rats overwhelmingly consumed sucrose water over regular water, whereas DFP exposed rats did not exhibit any such preference indicating anhedonia-like condition. Data expressed as mean \pm SEM, * $p < 0.05$, t-test, $n = 12-15$ rats.

Figure 3- EPM

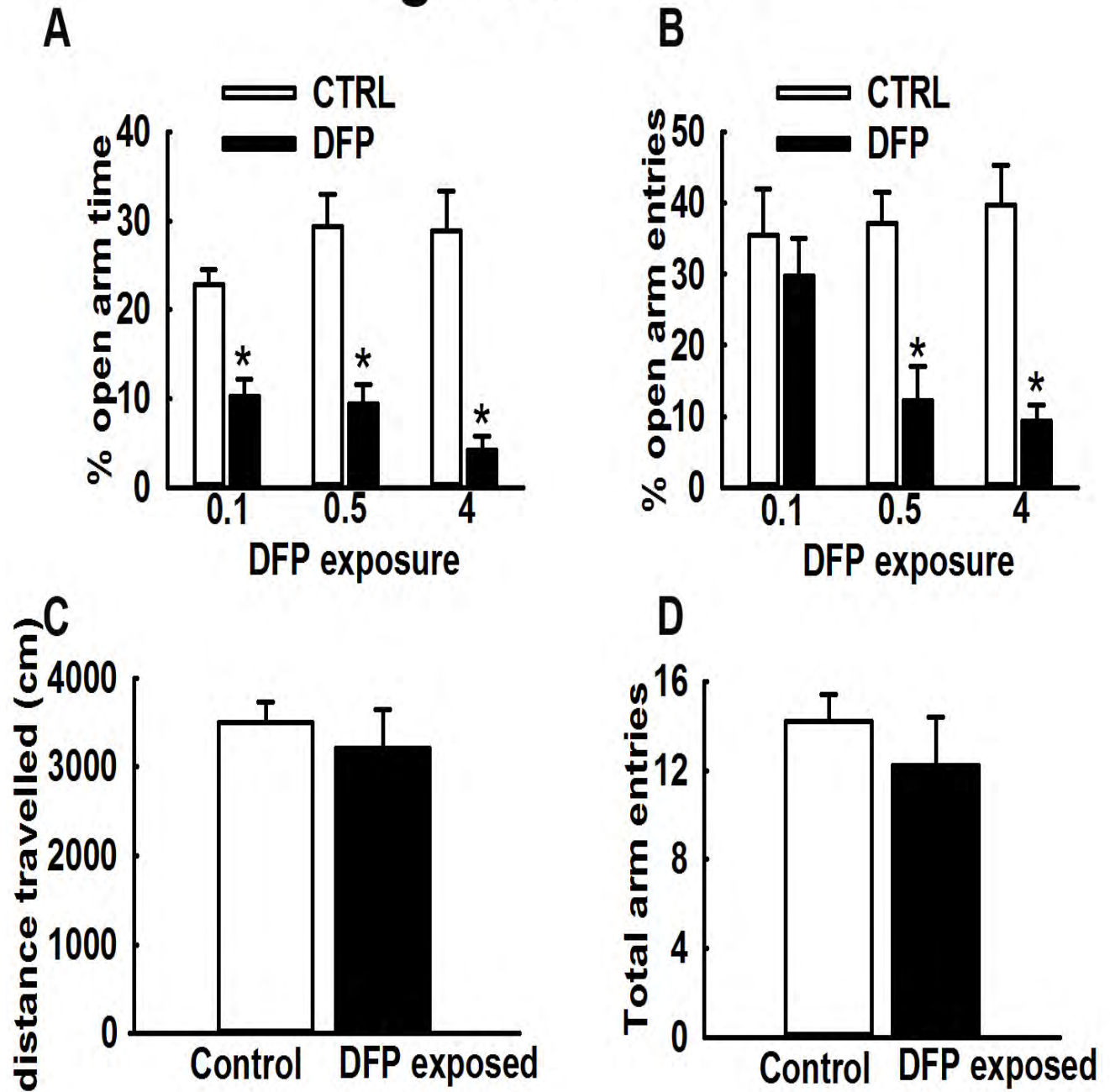


Figure 3. Increased anxiety in DFP exposed rats on EPM test. DFP exposed rats displayed significantly lower open arm time (A) and open arm entries (B) compared to age-matched control rats. No differences were observed in the distance travelled (C) and total arm entries (D) between the two-groups. Data expressed as mean \pm SEM, * $p < 0.05$, t-test, $n = 11$ rats.

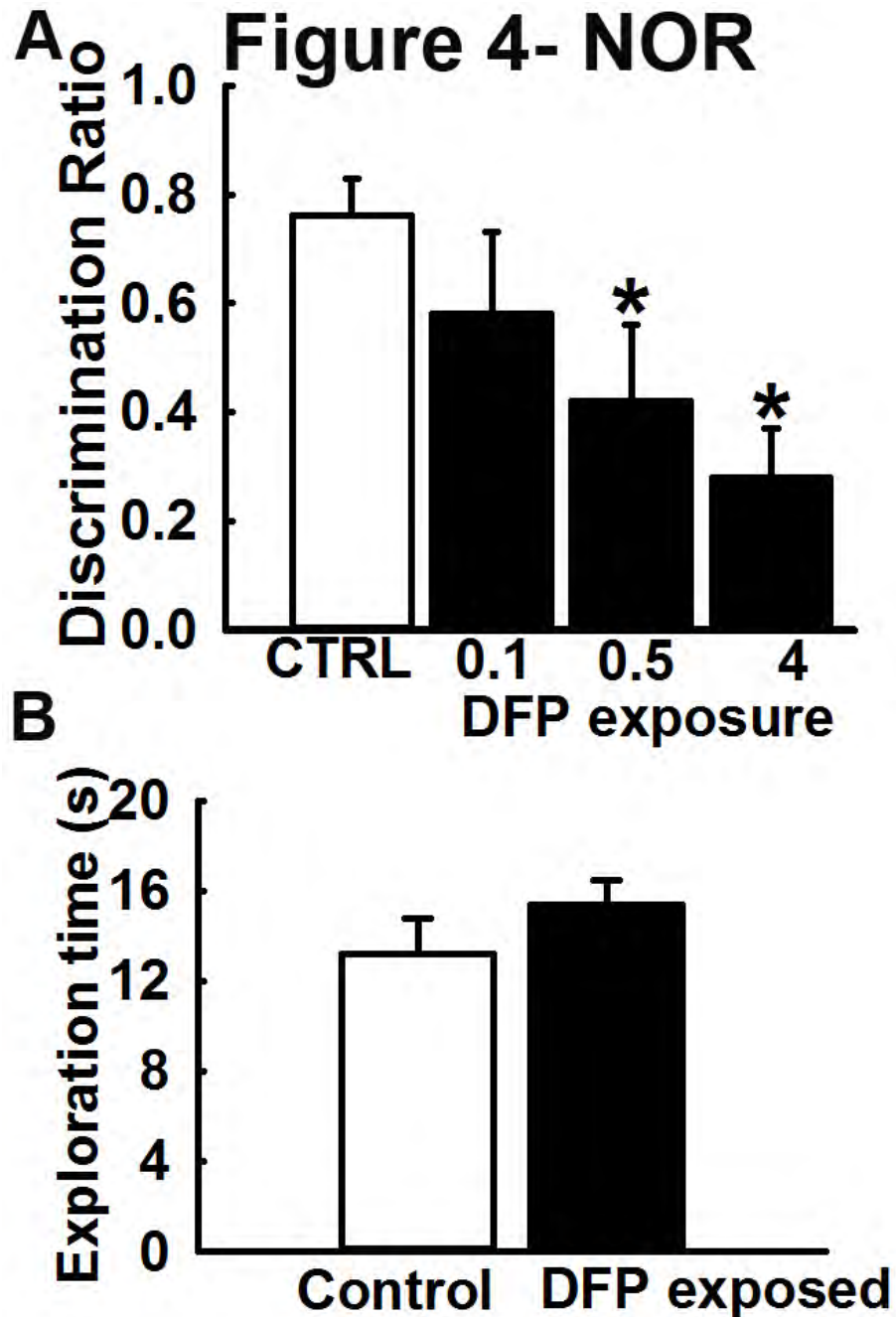
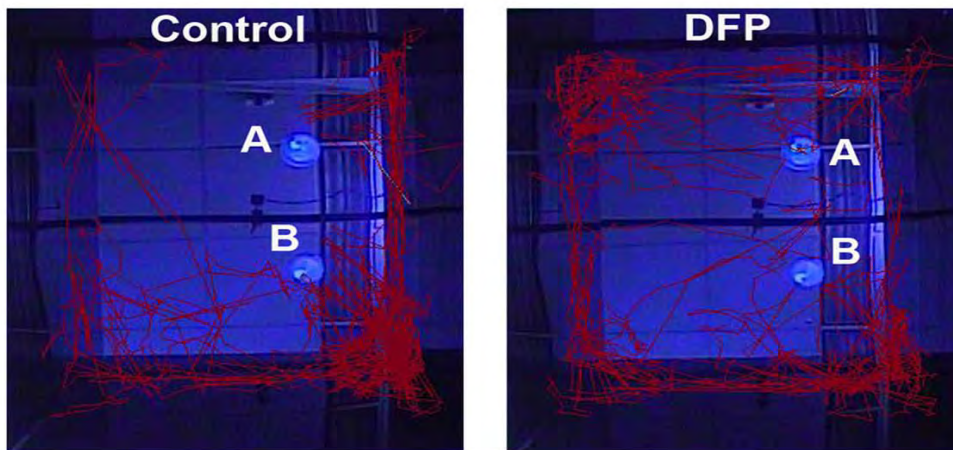


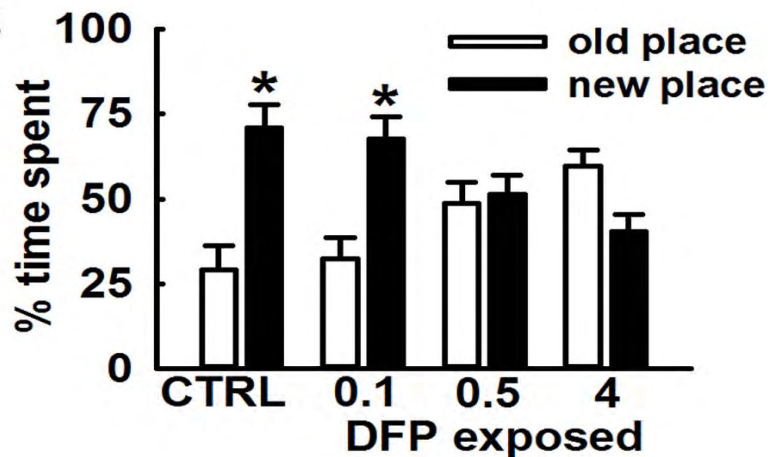
Figure 4. Impaired recognition memory in DFP exposed rats on NOR test. (A) DFP rats (0.5 and 4 mg/kg, s.c.) exhibited a negative discrimination ratio indicative of impaired recognition memory that was significantly lower compared to a positive discrimination ratio observed in age matched control rats. The low-dose DFP exposure group was not different from age-matched control rats. (B) No significant differences were observed in the exploration time during the identical object-sample phase test session between the two groups. Data expressed as mean \pm SEM, * p <0.05, t-test, n = 12-15 rats.

Figure 5- OLT

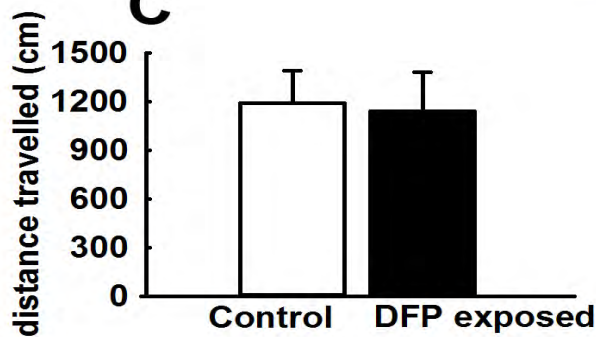
A



B



C



D

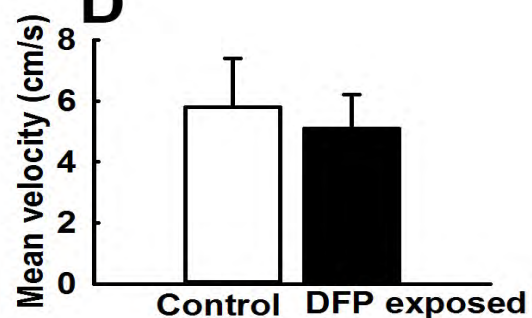


Figure 4. Impaired spatial memory in DFP exposed rats on OL test. (A, B) DFP exposed rats showed no preference for when the object was moved to new location-B indicative of impaired spatial memory that was significantly lower compared to the time spent by age matched control rats at the new location. No significant differences were observed in the distance travelled (C) and mean velocity (D) during the test session between the two groups. Data expressed as mean \pm SEM, * p <0.05, t-test, n = 15 rats.

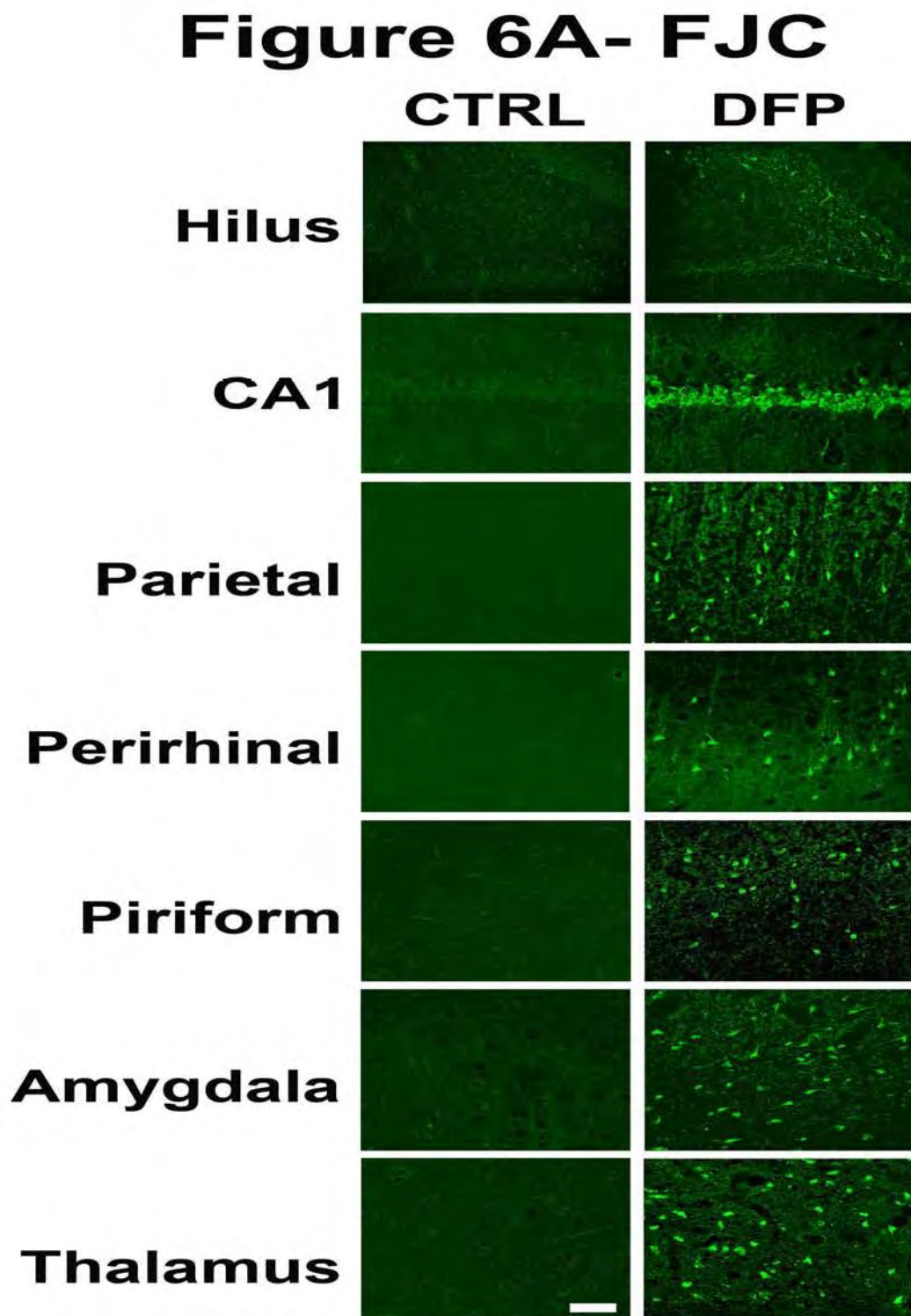


Figure 6A. High dose DFP induced neuronal injury. Representative photomicrographs of Fluoro-Jade C (FJC) staining in the (a) dentate gyrus-hilus region, (b) CA1, (c) parietal cortex, (d) peri-rhinal cortex, (e) piriform cortex, (f) amygdala, and (g) thalamus of a control rat (left panel) and DFP rat (4 mg/kg, s.c.) Scale bars, 50 μ m.

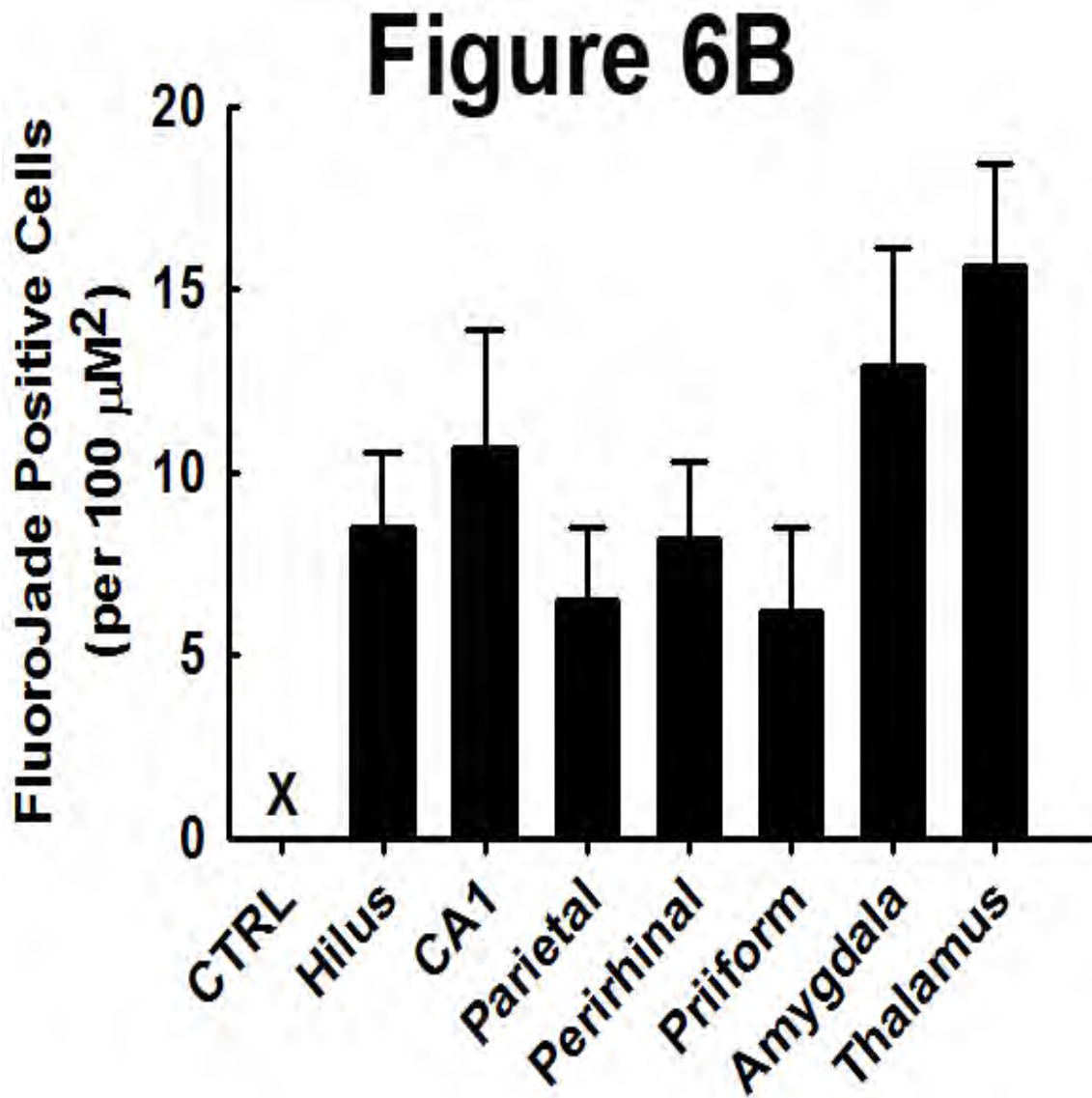


Figure 6B. Quantitative analyses of FJC labeling. FJC positive cells indicative of neuronal injury were observed in hilus, CA1, parietal cortex, perirhinal cortex, piriform cortex, amygdala, and thalamus high-dose POX rats. Control rats did not exhibit any FJC labeling.

Figure 6C

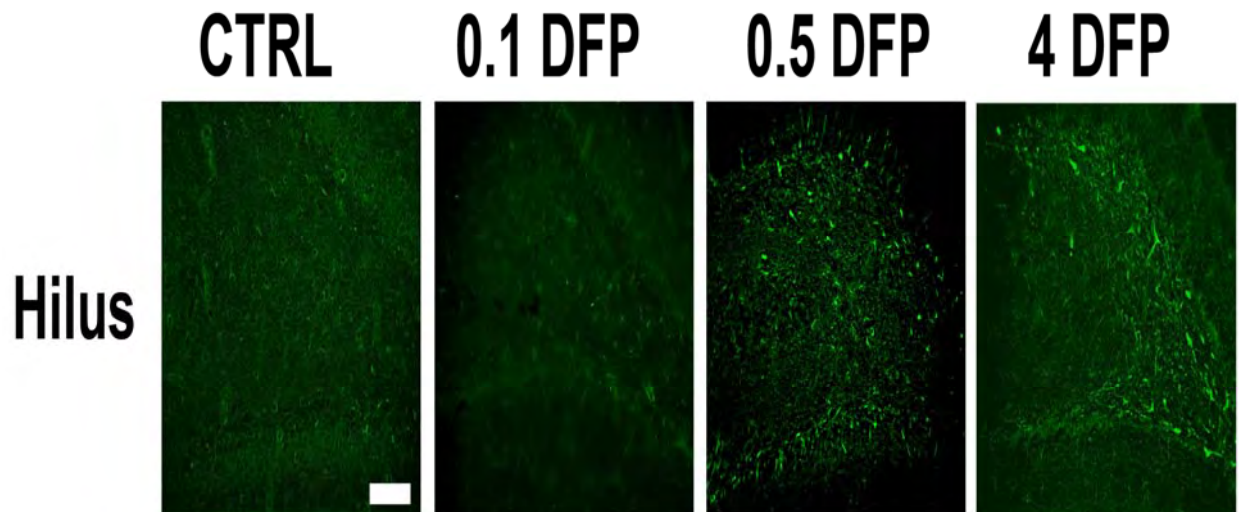


Figure 6C. DFP induced hippocampal neuronal injury. Representative photomicrographs of Fluoro-Jade C (FJC) staining in the dentate gyrus-hilus region of rats following vehicle, low, moderate and high dose DFP exposure. Scale bars, 50 μ m.

3.3. What opportunities for training and professional development has the project provided?

This project provided Dr. Kristin Phillips, a post-doctoral fellow, the opportunity to learn various rodent behavioral assays. When she joined my laboratory, she had experience in developing hypothermia protocols for brain injury treatment. This grant gave me an opportunity to train her in designing behavioral screening experiments, automating the data acquisition and the final analysis in order to study neuropsychiatric symptoms of depression, anxiety, and cognitive impairments. This grant has also given both of us professional development opportunities. Our abstract has been accepted for presentation at American Epilepsy Society's annual meeting. We will travel to Philadelphia to present our work and interact with other investigators and leaders in the field. Ms. Alaina Hudson, a high school senior from Fredericksburg, VA, underwent an unpaid internship in my laboratory during summer-2015. She observed rat DFP exposure protocols and various rodent behavioral assays and gained valuable research experience.

3.4 How were the results disseminated to communities of interest?

Our work on the Gulf War Illness project was recently highlighted in the VCU Neurology Department's newsletter "*Brain Waves*". This newsletter is communicated by the department to all the major academic medical centers in the country including Veterans Affairs Medical Centers and Neurology related medical establishments. This newsletter is attached in the appendices. In addition, we will be presenting our work to basic scientists and neurologists at the 2015 *American Epilepsy Society* annual meeting. Our work on model development is currently under-review at "*Neurotoxicology*". Upon acceptance, we will explore the open-access option for wider dissemination of the results to all the interested communities.

3.5 What do you plan to do during the next reporting period to accomplish the goals?

Having identified DFP exposure levels and demonstrated the presence of chronic depression, anxiety, and memory impairments following such exposures and concurrent damage in related brain area, we believe that we have established a rodent model of OP exposure that leads to the development of GWI related neurological morbidities. The goals for year-2 are thus to identify molecular mechanisms responsible for expression the of GWI psychiatric dysfunction. We will accomplish these goals by measuring intracellular Ca^{2+} levels using fluorescent microfluorimetry and estimate protein levels of components involved in the Ca^{2+} -induced Ca^{2+} release (CICR) machinery. The rationale for these studies is as follows:

Calcium is a major second messenger and plays a vital role in cellular signaling, in developing neuronal plasticity which controls behavior, and memory [36,37]. Thus, the levels of Ca^{2+} are tightly regulated by an intricate system of ion-channels, buffers, pumps and intracellular stores (ER). Brief elevations in Ca^{2+} levels are critical to cellular communication and long-term potentiation (learning and memory consolidation). However, our research and that of other investigators have demonstrated that sustained Ca^{2+} elevations particularly in the hippocampal region are detrimental to the cell and are implicated in many neurological disorders including Alzheimer's disease [38], Parkinson's disease [39], traumatic brain injury [40], aging [41], epilepsy [42] and stroke [43]. These neurological conditions are typically associated with cognitive deficits and other bio-behavioral disorders. The hippocampus plays a major role in the limbic system, is essential in memory functioning [21] and plays a major role in pathophysiology of depression [22]. Studies have shown hippocampal dysfunction in Gulf War veterans using

both imaging and neuropsychological testing [24-26]. OP-based animal models of GWI have also demonstrated hippocampal and striatal neuronal loss, inflammation, and reduced synaptic transmission underlying the expression of anxiety, mood and memory deficits [30-34]. Thus, hippocampus is an important brain area to investigate in GWI.

There has been recent evidence that the CICR system, principally consisting of the inositol-trisphosphate receptor (IP₃R) and the ryanodine receptor (RyR), plays a distinct role in memory processing and disease state [37,45-47]. The RyR-dependent Ca²⁺ release appears to aid the consolidation of labile memory into a persistent long-term memory trace while IP₃Rs are required during the formation of long-term memory [37]. The RyR can be activated via multiple mechanisms including the PKA dependent phosphorylation [48,49]. A permanent activation results in the development of “leaky” RyRs that raises [Ca²⁺]_i [48,50]. These phosphorylated RyRs have been implicated in stress-induced cognitive dysfunction [51]. Indeed, it has been recently shown that the RyR antagonist dantrolene significantly improves cognition in a murine model of Alzheimer's disease [52]. Similarly, pharmacological blockade of the intracellular Ca²⁺ release using both the IP₃R and RyR antagonist has been demonstrated to produce anti-depressant effect in forced swim test [53], a widely used rodent model of depression. Furthermore, knockdown of RyR subtypes in the brain also exhibited an anti-depressant effect [54]. Inhibition of IP₃R and PLCγ, which is responsible for producing IP₃ [55], has been reported to produce anti-depressant effect in rodents [56]. Moreover, there is new evidence that levetiracetam which we have reported to inhibit both the IP₃ and RyR mediated Ca²⁺ release in hippocampal neurons [57] also produces anti-depressant effect in forced swim test [58], has anxiolytic profile in the elevated plus maze test [59] and improves memory following traumatic brain injury in rats [60]. We have recently shown that the CICR system in hippocampal neurons was responsible for maintaining the long-lasting Ca²⁺ plateau after brain injury such that treatment with the CICR RyR antagonist, dantrolene, abolished the Ca²⁺ plateau and prevented the development of spontaneous recurrent epileptiform discharges in a hippocampal neuronal culture model of epilepsy [44]. Thus, there is mounting evidence that the hippocampal CICR Ca²⁺ signaling system is critical in mood and memory processing and that disturbance in this cascade produces depressive symptoms and memory impairments, and inhibiting this system with pharmacological or genetic manipulation affords relief from the symptoms of the affective disorders. At present, the role of CICR signaling system in the development of depression and cognitive impairments in GWI is unknown. Thus, this application will take an innovative approach and investigate for the first time the role of CICR signaling in causing depression and cognitive deficits in a rodent model of GWI developed in our laboratory.

Acute Isolation of Hippocampal Neurons for Ca²⁺ imaging:

Hippocampal CA1 neurons will be acutely isolated from intact animals and subjected to evaluation of [Ca²⁺]_i levels and CICR activity employing techniques routinely used in this laboratory. At the given time points, animals will be anesthetized with isoflurane and sacrificed immediately by decapitation, brains rapidly removed, hippocampal slices prepared and neurons acutely isolated and affixed to imaging chambers using cellular adhesive Cell-Tak™ (BD-Biosciences). Neurons will be loaded with the fluorescent Ca²⁺ indicator, Fura-2AM, to measure [Ca²⁺]_i and evaluated using micro-fluorimetry with an Olympus fluorescent microscope. Under the microscope a visual field containing 2-3 hippocampal neurons (identified morphologically) will be located. A minimum of 10 neurons will be recorded for both stimulations. A Ca²⁺

calibration curve to compare 340/380 fluorescence ratios to $[Ca^{2+}]_i$ concentrations using the equation: $[Ca^{2+}]_i = K_d [R - R_{min} / R_{max} - R] \times [Sf_2 / Sb_2]$ will be used.

Western Blot Analysis of RyR-p, IP₃R, PKA and PLC γ Expression:

Animals will be sacrificed and hippocampal tissue processed for Western blot studies at each time point following DFP exposure using standard procedures established in the literature and routinely performed in this laboratory to measure proteins in control and DFP hippocampi. From each experimental animal, hippocampal homogenates will be prepared. Quantitation of the amount of RyR-p, IP₃-R, PKA and PLC γ per mg protein in each sample will be performed. Antibody specificity will be established using blocking peptides and no antibody controls. Internal and external standards to control for loading and sample variability will be used.

4. Impact

The Research Advisory Committee on Gulf War Veterans Illness has strongly implicated exposure to OP nerve agents as leading cause for GWI. In this first year of grant, my laboratory has successfully developed a rodent model of OP exposure using nerve agent surrogate, DFP that exhibits chronic GWI symptoms reflecting the morbidities observed in Gulf War veterans, including the development of anxiety, depression and cognitive deficits months after initial OP exposure. We have also identified neuronal damage in the same brain areas that have been reported to be compromised in GW veterans in clinical, functional and imaging studies.

4.1 What was the impact on the development of the principal discipline(s) of the project?

We have outlined the details of model development in the section-3. A manuscript describing this research effort is currently under peer-review. We believe that once our manuscript is vetted and published it will represent one of the first evidence that nerve agent exposure in the absence of other confounding factors such as stress, pyridostigmine tablets or other insecticides can produce neurological morbidities similar to GWI. This will have a major impact on the lives of veterans suffering from GWI by allowing investigators a novel model of GWI symptoms to identify molecular bases of GWI in search of providing GW veterans with additional, effective therapeutic options.

4.2 What was the impact on other disciplines?

Exposure to OP agents either occupational, accidental, or terrorism-related is a legitimate concern. Our work involving model development in year-1 has the capability to also serve as a rodent model of chronic OP exposure in the civilian population.

4.3 What was the impact on technology transfer?

“Nothing to report”

4.4 What was the impact on society beyond science and technology?

“Nothing to report”

5. Changes/ Problems:

We did not encounter any major problems during this reporting period. We did encounter delays in acquiring DFP from the supplier at the beginning of the grant period, however we were able to make up for the lost time by the end of the year.

6. Products

6.1 Publications, conference papers, and presentations

6.1.1 Journal publications:

Phillips KF and Deshpande LS. Repeated low-dose organophosphate DFP exposure leads to the development of depression and cognitive impairment in a rat model of Gulf War Illness. Neurotoxicology (under-review. MS#. NEUTOX-D-15-00277). Federal support acknowledged.

6.1.2 Other publications, conference papers, and presentations

Phillips KF, Deshpande LS, Huang BA and DeLorenzo RJ. Behavioral depression and memory impairment following organophosphate diisopropyl fluorophosphate induced status epilepticus in rats. Abstract No 2.040, 2015, American Epilepsy Society Annual Meeting, www.aesnet.org (accepted),

7. Participants & Other Collaborating Organizations

7.1 What individuals have worked on the project?

Name	Laxmikant Deshpande	Kristin Phillips	Robert Blair
Project Role	PI	Post-Doc Fellow	Investigator
Research Identifier	orcid.org/0000-0003-1491-1561		
eRA commons:	DESHPANDELS	KPHILLIPS5	REBLAIR
Nearest person month worked	7.2	12	2.5
Contribution to the project	DFP exposure studies, behavioral assays, GWI model development, data analysis, manuscript writing and submission	Behavioral analysis of DFP exposed rats, Specimen preparation for histological procedures	Histological screening for assessing neuronal damage
Funding support	DOD, NINDS	DOD	DOD, NINDS

7.2 Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

“Nothing to report”

7.3 What other organizations were involved as partners?

“Nothing to report”

8. Special reporting requirements

“Not applicable”

9. Appendices

9.1 Bibliography

9.2 “Brain Waves” Newsletter

9.3 2015 AES abstract

9.1 Bibliography

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9.2 Department of Neurology Newsletter: Brain Waves

Resident and Fellow Graduation

We will say goodbye to our senior residents and fellows at a graduation celebration on June 4th, 2015



Valerie Klats, a 4th year medical student, received the American Academy of Neurology Medical Student Prize for Excellence. This honor is awarded to a student who has outstanding evaluations and recommendations from faculty and residents and who shows the most promise for a career in neurology. Congratulations to Valerie!

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Spotlight on Basic Science Research Laxmikant Deshpande, M.Pharm, Ph.D.



Congratulations to:

Laxmikant Deshpande ("LD") who was recently awarded a Department of Defense Grant: "Novel Therapeutic Approaches for the Treatment of Depression and Cognitive Deficits in a Rodent Model of Gulf War Veterans' Illness"

The First Gulf War that ended Saddam Hussein's Iraqi occupation of Kuwait is considered by many as a textbook example of military precision and US super power. Less than 200 battle related deaths occurred during "Operation Desert Storm". Unknowingly, this military success was associated with a major health disaster for the returning troops. By some estimates, one in three Gulf War veterans (approximately 250,000 veterans) is suffering from a cryptic illness commonly known as Gulf War Syndrome or Chronic Multi-symptom Illness. Amongst many probable causes, exposure to organophosphate pesticides and low-dose exposure to the nerve gas Sarin are considered to be one of the primary causes of Gulf War Illness.

Through a research grant from the Department of Defense, Congressionally Directed Medical Research Program, our laboratory is developing a rat model that mimics various organophosphate exposure levels and has identified chronic neurological problems such as anxiety, depression and memory impairments similar to Gulf War Veterans. Using this model of Gulf War Illness morbidities, our laboratory has identified alterations in the intracellular handling of calcium ions. We aim to decipher molecular mechanisms underlying this neuronal abnormality. Our translational work is focused on identifying drugs that would target these alterations and effectively treat Gulf War Illness symptoms. This research could provide proof-of-concept studies that would fast-track already approved FDA drugs in the clinical trials for the treatment of Gulf War Illness symptoms.

Our laboratory also collaborates with Dr. Robert DeLorenzo on an NIH CounterACT project. This is a trans-NIH effort designed to enhance the nation's diagnostic and treatment response capabilities during a chemical emergency- either by act of terrorism, industrial accident or natural disaster. We have developed rat survival models of lethal organophosphate intoxication and are using them to decipher molecular mechanisms underlying chronic effects of organophosphate toxicity. We are also screening novel countermeasure agents to reduce mortality and morbidity associated with lethal organophosphate exposure.

This work is being carried out by Dr. Laxmikant S. Deshpande and Dr. Kristin Phillips in the Department of Neurology.

9.3 AES Abstract Details

Control ID: 2325771

Abstract No. 2.040

Presentation type: 1. Standard poster

Title: Behavioral Depression and Memory Impairment Following Organophosphate Diisopropyl fluorophosphate Induced Status Epilepticus in Rats

Authors: Phillips, Kristin¹ ; Deshpande, Laxmikant¹ ; Huang, Beverly¹ ; Delorenzo, Robert¹

Institutions: 1. Neurology, Virginia Commonwealth University, Richmond, VA, USA.

Current topic: 1. Translational Research: 1b. Animal or Computational Models

Abstract body:

Rationale: Organophosphate (OP) compounds include pesticides and nerve agents that elicit lethal toxicity by inhibiting acetylcholinesterase (AChE) which leads to an acute cholinergic syndrome that evolves into status epilepticus (SE). There is a growing concern that OP agents could be used to cause mass civilian casualties. Similar to the survivors of SE, OP toxicity is associated with neurobehavioral deficits including mood changes, depression, and memory impairments. In this study we investigated whether animals surviving lethal OP exposure exhibited long-term neurological impairments, using diisopropyl fluorophosphate (DFP), an OP agent used in civilian laboratories to mimic effects of nerve agent exposure.

Methods: Male Sprague-Dawley rats (250-300g) were injected with DFP (4 mg/kg, s.c). One minute following DFP injection, rats were injected with atropine sulfate (2 mg/kg, i.p) and 2-PAM (25 mg/kg, i.m). Rats exhibited cholinergic crisis, including the occurrence of status epilepticus. Seizures were stopped with three injections of diazepam (5 mg/kg, i.p) plus 2-PAM at 1, 3 and 5-hr following the onset of SE. Approximately 4-months following DFP exposure the rats were screened for depressive symptoms using forced swim test (FST), sucrose preference test (SPT) and elevated plus maze (EPM). Cognitive deficits were investigated using the novel object recognition test (NORT).

Results: DFP-SE rats subjected to FST exhibited increased immobility time ($83.03 \pm 8.5s$, $n = 5$) indicative of a despair-like state that was significantly higher than age matched controls ($35.46 \pm 3.8s$, $n = 5$, $p < 0.05$). In the SPT, DFP rats consumed significantly less sucrose water indicating anhedonia-like condition ($58.40 \pm 5.9\%$ sucrose preference in DFP rats Vs $82.66 \pm 6.5\%$ in control rats). DFP rats also displayed increased anxiety as characterized by significantly lower performance in the open arm of the EPM (time in open arm: $18.9 \pm 4.5\%$ in controls Vs $4.2 \pm 1.5\%$ in DFP rats). In NORT, DFP SE rats exhibited a discrimination ratio of 0.28 ± 0.09 indicative of impaired recognition memory that was significantly lower compared to age matched control rats (0.76 ± 0.07 , $n = 5$, $p < 0.05$).

Conclusions: Here we observed depression-like symptoms and cognitive deficits in rats surviving severe OP exposure. Evidence from the civilian population indicates that repeated exposure to OP insecticides or a single acute exposure to nerve agents can lead to chronic neurological morbidities. Approximately 35% of soldiers deployed in the 1991 Persian Gulf War suffer from chronic multi-symptom illnesses characterized by depression and cognitive deficits, which is thought to be due to OP/ nerve agent exposure during deployment. SE is also known to produce cognitive deficits by affecting the hippocampus and changing neuronal networks in the brain. This DFP model mimics both the acute hyper-cholinergic response and exhibits chronic behavioral impairments and cognitive deficits. It is being used to identify molecular mechanisms and screen novel pharmacological agents for effective treatment of these co-morbid disorders.